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Mutations in the E2 Glycoprotein of Venezuelan Equine Encephalitis Virus Confer Heparan Sulfate Interaction, Low Morbidity, and Rapid Clearance from Blood of Mice

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The arbovirus, Venezuelan equine encephalitis virus (VEE), causes disease in humans and equines during periodic outbreaks. A murine model, which closely mimics the encephalitic form of the disease, was used to study mechanisms of attenuation. Molecularly cloned VEE viruses were used: a virulent, epizootic, parental virus and eight site-specific glycoprotein mutants derived from the parental virus. Four of these mutants were selected *in vitro* for rapid binding and penetration, resulting in positive charge changes in the E2 glycoprotein from glutamic acid or threonine to lysine (N. L. Davis, N. Powell, G. F. Greenwald, L. V. Willis, B. J. Johnson, J. F. Smith, and R. E. Johnston, *Virology* 183, 20–31, 1991). Tissue culture adaptation also selected for the ability to bind heparan sulfate as evidenced by inhibition of plaque formation by heparin, decreased infectivity for CHO cells deficient for heparan sulfate, and tight binding to heparin–agarose beads. In contrast, the parental virus and three other mutants did not use heparan sulfate as a receptor. All eight mutants were partially or completely attenuated with respect to mortality in adult mice after a subcutaneous inoculation, and the five mutants that interacted with heparan sulfate *in vitro* had low morbidity (0–50%). These same five mutants were cleared rapidly from the blood after an intravenous inoculation. In contrast, the parental virus and the other three mutants were cleared very slowly. In summary, the five VEE viruses that contain tissue-culture-selected mutations interacted with cell surface heparan sulfate, and this interaction correlated with low morbidity and rapid clearance from the blood. We propose that one mechanism of attenuation is rapid viral clearance *in vivo* due to binding of the virus to ubiquitous heparan sulfate.

INTRODUCTION

Venezuelan equine encephalitis virus (VEE), an alphavirus in the togavirus family, is a single-stranded, positive-sense RNA virus. The alphavirus genome is encapsidated by the capsid protein and surrounded by a highly structured envelope (Paredes *et al.,* 1993) consisting of two membrane glycoproteins, E1 and E2 (Rice and Strauss, 1982). E2 is the putative viral attachment protein for VEE (Roehrig *et al.,* 1988) and, thus, interacts with the host cell.

The interaction of virus with its host cell receptor is one of the important factors dictating host and tissue tropism. VEE and other arboviruses cycle between mosquitoes and mammals in nature. VEE therefore has evolved to use either a universally conserved receptor or multiple receptors in its disparate hosts. Furthermore, VEE has evolved to infect a variety of tissues. In the mosquito, VEE infects the mesenteron, fat body, nerves, brain, and salivary glands (Weaver, 1986). In vertebrate hosts, VEE is both lymphotropic and neurotropic (Victor *et al.,* 1956; Gleiser *et al.,* 1961; Jahrling and Scherer, 1973a; de la Monte *et al.,* 1985). The mammalian receptor for VEE remains unknown, but a laminin-binding protein was identified as the putative host cell receptor for VEE in mosquito cell culture (Ludwig *et al.,* 1996). A similar molecule on mammalian cells, the high-affinity laminin receptor, was suggested as a receptor for another alphavirus, Sindbis virus (SIN) (Wang *et al.,* 1992). SIN can also interact with heparan sulfate (HS) on mammalian cells (Mastromarino *et al.,* 1991; Byrnes and Griffin, 1998; Klimstra *et al.,* 1998). However, Klimstra *et al.* (Klimstra *et al.,* 1998) demonstrated for SIN that HS binding is a cell culture adaptation and is correlated with attenuation in mice. Thus, we postulated that HS also may play a role in VEE binding and pathogenesis *in vivo.*

Heparan sulfate is a glycosaminoglycan (GAG). GAGs consist of repeating disaccharide units that are heterogenously sulfated, and they covalently bind to protein cores to form proteoglycans. Proteoglycans are ubiquitous on the cell surface and in the extracellular matrix. One function of proteoglycans is to bind a variety of ligands, e.g., laminin, fibroblast growth factor, antithrombin III, and superoxide dismutase (Zhou *et al.,* 1992). The most common GAGs are heparan sulfate, chondroitin sulfate, and dermatan sulfate (Kjellen and Lindahl, 1991).

The first virus shown to interact with cell surface HS was herpes simplex virus (WuDunn and Spear, 1989). Several other herpesviruses also interact with HS, including pseudorabies virus, cytomegalovirus, and bovine

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TABLE 1

Amino Acid Changes in the Glycoprotein Sequences of VEE Mutants

VEE clone[a]	E3	E2						E1	
	56–59	3	4	76	116	120	209	239	253	272
V3000	RKRR	E	E	E	K	T	E	I	F	A
V3010	RKRR	E	E	K	K	T	E	I	F	A
V3012	RKRR	E	E	E	K	K	E	I	F	A
V3014	RKRR	E	E	E	K	T	K	N	F	T
V3026	RKRR	K	K	E	K	T	E	I	F	A
V3032	RKRR	E	E	E	K	T	K	I	F	A
V3034	RKRR	E	E	E	K	T	E	I	F	T
V3526	Δ[b]	E	E	E	K	T	E	I	S	A
V3533	RKRR	E	E	K	E	T	E	I	F	A

[a] All clones are isogenic except at the noted loci.

[b] Δ, deletion of the furin cleavage site (amino acids 56 to 59 of E3), resulting in an E3–E2 fusion protein.

herpesvirus types 1 and 4 (Mettenleiter *et al.,* 1990; Okazaki *et al.,* 1991; Neyts *et al.,* 1992; Kari and Gehrz, 1992; Vanderplasschen *et al.,* 1993). Recent studies have shown that many other viruses interact with HS in cell culture, but the functional significance of HS binding for non-cell culture adapted viruses has not always been determined. These viruses include adenoassociated virus (AAV), vaccinia virus, human papillomavirus, human immunodeficiency virus (HIV), foot-and-mouth disease virus (FMDV), respiratory syncytial virus, porcine reproductive and respiratory syndrome virus, equine arteritis virus, and dengue virus (Patel *et al.,* 1993; Jackson *et al.,* 1996; Asagoe *et al.,* 1997; Chen *et al.,* 1997; Jusa *et al.,* 1997; Krusat and Streckert, 1997; Chung *et al.,* 1998; Summerford and Samulski, 1998; Joyce *et al.,* 1999).

Many of these viruses use other cell receptors in addition to HS. Herpesviruses bind a second receptor (Montgomery *et al.,* 1996; Warner *et al.,* 1998; Geraghty *et al.,* 1998). HIV uses CD4 and a chemokine coreceptor (Dalgleish *et al.,* 1984; Berger *et al.,* 1999), and FMDV uses integrin as its receptor (Neff *et al.,* 1998). Alternative cell receptors have recently been postulated for AAV (Qing *et al.,* 1999; Summerford *et al.,* 1999). Several authors have proposed a model where initial binding to HS is followed by binding and entry via a coreceptor (Patel *et al.,* 1993; Spear, 1993; Summerford *et al.,* 1999). In other viral systems, HS may serve as an alternate receptor, and its use is dependent on the cell type and viral strain. Ibrahim *et al.* (Ibrahim *et al.,* 1999) found that HIV uses HS on cell lines, but not on primary lymphocytes. HS usage is a tissue culture adaptation for FMDV (Sa-Carvalho *et al.,* 1997; Baranowski *et al.,* 1998; Escarmis *et al.,* 1998; Neff *et al.,* 1998) and SIN (Klimstra *et al.,* 1998). In other words, the wild-type viruses do not bind HS, but use an alternate receptor exclusive of GAG binding. Furthermore, adaptation to tissue culture and HS usage is correlated with attenuation for FMDV (Sa-Carvalho *et al.,* 1997; Neff *et al.,* 1998) and SIN (Klimstra *et al.,* 1998, 1999; Byrnes and Griffin, 2000).

Our laboratory has developed a panel of VEE mutants, which were selected for rapid binding and penetration in tissue culture (Johnston and Smith, 1988; Davis *et al.,* 1991). The SIN mutants that were studied by Klimstra *et al.* (Klimstra *et al.,* 1998) were similarly selected and were found to use HS as a receptor; therefore, we hypothesized that the VEE mutants would also use HS. In this study, we found that the VEE tissue-culture-adapted mutants, which have a net positive charge change in the E2 glycoprotein, interacted with cell surface HS. In contrast, the wild-type virus and three other VEE glycoprotein mutants did not. Furthermore, HS interaction *in vitro* correlated with low morbidity *in vivo* and rapid clearance of virus from the blood of mice, suggesting a mechanism of attenuation.

RESULTS

The genotypes of the viruses used in this study are shown in Table 1. All of the mutants are attenuated relative to the parental virus (Table 2). Although the mortality for V3034 was not statistically different from that of V3000 for this experiment, the mortality was statistically different for a previous experiment ($P < 0.001$) (Grieder *et al.,* 1995). Furthermore, we have inoculated over 80 mice with V3000 using the same experimental conditions and have not observed a survivor (Davis *et al.,* 1995; Grieder *et al.,* 1995; Aronson *et al.,* 2000; R. E. Johnston *et al.,* unpublished observations), supporting our conclusion that V3034 is attenuated.

Four of the VEE mutants (V3010, V3012, V3014, and V3026) were selected on BHK cells for rapid binding and penetration (Johnston and Smith, 1988). All four of these viruses have an amino acid change in the E2 glycoprotein to a positively charged lysine (Davis *et al.,* 1991). The mutant V3026 has two contiguous codons with both

TABLE 2

Morbidity and Mortality of VEE Viruses after SC Inoculation

VEE clone	Morbidity (No. sick/No. mice)	Mortality (No. dead/No. mice)
V3000	8/8	8/8
V3010	0/8	0/8*
V3012	4/8	2/8**
V3014	0/8	0/8*
V3026	0/8	0/8*
V3032	2/8	1/8***
V3034	7/8	5/8†
V3526	0/8	0/8*
V3533	8/8	0/8*

Note. Adult mice were inoculated subcutaneously in the left rear footpad with 10^3 PFU of virus. Results of a one-sided Fisher's exact test comparing the mortality of each group to the parental virus, V3000, were $*P < 0.0001$, $**P = 0.0035$, $***P = 0.0007$, $\dagger P = 0.1$.

TABLE 3

Glycosaminoglycan Inhibition of Viral Infectivity for BHK Cells

VEE clone	GAG IC_{50} (μg/ml)[a]		
	Heparin	Dextran sulfate	Chondroitin sulfate B
V3000	>1000	>1000	>1000
V3010	3	45	350
V3012	75	30	>1000
V3014	20	45	400
V3026	3	200	300
V3032	15	500	200
V3034	>1000	>1000	>1000
V3526	350	500	>1000
V3533	>1000	>1000	>1000

Note. Plaque assays were performed with increasing amounts of GAG. The data are representative of three independent assays.

[a] Average of three wells.

changed to lysine. The mutant V3014 has three mutations; two mutations (E2 209 and E1 272) are attenuating, and one mutation (E2 239) has no effect on virulence (Grieder *et al.,* 1995). Each attenuating mutation also is represented alone as a single mutant (V3032 and V3034). Another tissue-culture-selected mutant, V3010, is limited in spread beyond the draining lymph node after SC inoculation of adult mice (Grieder *et al.,* 1995). Aronson *et al.* (Aronson *et al.,* 2000) selected for revertants of V3010 in adult mice, and the mutant V3533 is a second-site revertant that spreads beyond the draining lymph node, but remains attenuated with respect to mortality. This reversion restores the net charge of E2.

The mutant V3526 has a lethal deletion in the furin cleavage site at the C-terminus of E3 paired with a mutation in E1 that restores infectivity for BHK cells. In contrast to the parental virus, V3000, for which E3 is fully cleaved from E2, the V3526 construct results in an E3–E2 fusion protein incorporated into an infectious virion (Davis *et al.,* 1995). The other seven mutants listed in Table 1 had the parental phenotype of complete cleavage of E3 from E2 (data not shown).

Inhibition of VEE infectivity by heparin

The tissue-culture-selected mutants are altered in their interaction with the cell surface (Johnston and Smith, 1988). Since all the mutants have amino acid changes in E2 to a lysine (Davis *et al.,* 1991), one potential explanation for the *in vitro* phenotype is an interaction of the basic amino acid(s) in E2 with negatively charged GAGs on the cell surface. Competition assays were performed to determine the infectivity of each virus in the presence of heparin (a highly sulfated, secreted form of HS), chondroitin sulfate B, and dextran sulfate. All five viruses with tissue-culture-selected mutations (V3010, V3012, V3014, V3026, and V3032) were inhibited by GAGs. The IC_{50} was lowest for heparin (Table 3), and the inhibition was dose dependent (Fig. 1). In contrast, the parental virus and the other three mutants were not inhibited (V3000, V3034, and V3533) or were inhibited only at high concentrations (V3526) (Table 3). Of the two attenuating mutations of V3014, only the lysine at position 209 of E2 (V3032) was associated with heparin sensitivity. Furthermore, the mutant V3010 was sensitive to heparin, but its second-site revertant (V3533) was resistant like the parental virus (Fig. 1).

Infectivity of VEE virus for GAG-deficient cells

The infectivity of five VEE mutants was reduced in the presence of soluble heparin, suggesting that these viruses interact with cell surface GAGs. Alternatively, soluble heparin might coat the virus and sterically prevent

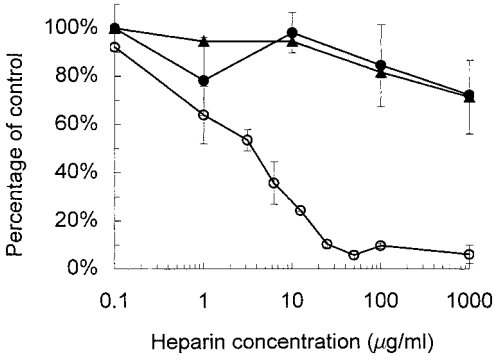

FIG. 1. The effect of heparin on VEE virus infectivity. BHK cells were infected with V3000 (▲), V3010 (○), or V3533 (●) in the presence of variable concentrations of heparin. The percentage of no treatment was determined as follows: (the number of viral plaques at the designated heparin concentration/the number of viral plaques without heparin) × 100%. Each data point is the average of three replicates ± SD. The data are representative of three independent assays.

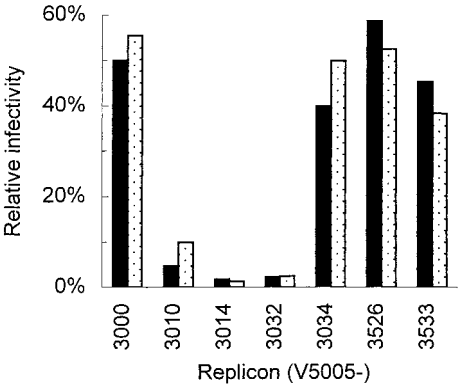

FIG. 2. The relative infectivity of VRP for GAG-deficient CHO cells. The cells were infected with VRP containing the GFP-expressing replicon (V5005) coated with the indicated glycoproteins (e.g., 3000). Solid bars represent the infectivity for pgsA-745 (deficient in all GAGs), and shaded bars represent the infectivity for pgsD-677 (deficient in HS). The relative infectivity was calculated as follows: (the number of cells infected for the indicated cell line/the number cells infected for the parental CHO-K1 cells) × 100%. The data are representative of two independent assays.

the virus from binding another receptor. To confirm that the viruses were interacting with cell surface GAGs, we tested their infectivity on GAG-deficient cells derived from CHO-K1 cells. VEE replicon particles (VRP) (Pushko *et al.,* 1997), which undergo only one round of replication, were used in these assays. These particles contain the same replicon genome, but are coated with the different envelope glycoproteins corresponding to the various VEE mutants. The VRPs with mutant glycoproteins resistant to heparin (V3000, V3034, V3526, and V3533) had an approximately 2-fold decrease in infectivity for the GAG-deficient cells compared to the parental CHO-K1 cells (Fig. 2). In contrast, VRPs from viruses that were inhibited by heparin (V3010, V3014, and V3032) had a 10- to 100-fold decrease in infectivity. The reduced infectivity of these VRPs occurred on both cell lines: pgsA-745, which are deficient in all GAGs (Esko *et al.,* 1985), and pgsD-677, which have increased levels of chondroitin sulfate, but are deficient in heparan sulfate (Lidholt *et al.,* 1992). Therefore, these results suggest that the tissue-culture-adapted viruses interacted with cell surface heparan sulfate. Furthermore, the use of VRP ensured that differences in infectivity were due to an early virus–cell interaction and were not due to differences in viral replication, maturation, or spread. Again, the second-site revertant, V3533, had the parental phenotype. Note that two of the viruses (V3012 and V3026) were not tested in this assay.

Binding of VEE viruses to immobilized heparin

To confirm that VEE viruses can bind GAGs, radiolabeled viruses were tested for their ability to bind to heparin–agarose beads. All nine viruses bound to the immobilized heparin to varying degrees (Fig. 3). There was no consistent correlation between the degree of binding and the putative interaction with cell surface heparan sulfate. There are at least two possible explanations for the lack of correlation: (1) the heparin–agarose bead displays a very dense group of negatively charged molecules, allowing the positively charged E2 (France *et al.,* 1979) to bind; (2) the radiolabeled viruses were passaged once in tissue culture; therefore, these populations may contain a subset of tissue-culture-selected variants that bind variably to heparin. Such mutants arise very early during passage of SIN on BHK cells (Klimstra *et al.,* 1998).

To distinguish the relative strength of heparin–agarose binding, four of the viruses were eluted from the heparin–agarose beads with increasing NaCl concentration. V3000 and V3533, which were heparin resistant in the competition assays, showed peak elution at 200 mM NaCl. In contrast, V3012 and V3032, which were heparin sensitive and interacted with cell surface heparan sulfate, showed peak elution at 350 and 450 mM NaCl, respectively (data not shown), suggesting substantially stronger HS binding.

In vivo phenotype of VEE mutants

The heparan sulfate interaction observed *in vitro* correlated with the *in vivo* phenotype of the viruses. The VEE mutants were partially or completely attenuated in adult mice after SC inoculation (Table 2). All five viruses that interacted with cell surface heparan sulfate (V3010, V3012, V3014, V3026, and V3032) resulted in low morbidity (0–50%). These data suggested that the heparan sulfate interaction influenced attenuation. Since HS is ubiquitous on mammalian cell surfaces, we postulated that the interaction of virus with HS would result in rapid clearance from the blood.

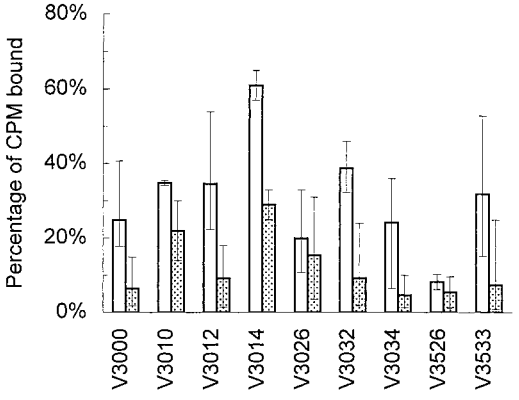

FIG. 3. The binding of radiolabeled virus to immobilized heparin. The open bars represent the binding to heparin–agarose beads, and the shaded bars represent the binding to BSA–agarose beads. Each bar represents the average for two to seven independent assays with bars representing high and low values for the individual assays. CPM is counts per minute of radioactivity.

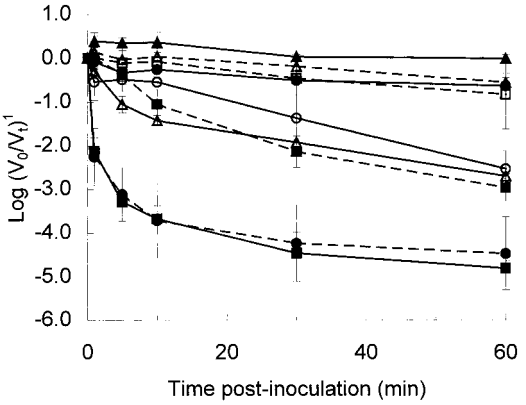

FIG. 4. The clearance of VEE virus from serum after an intravenous (IV) inoculation. [1]V_0 = virus concentration in serum (PFU/ml) at time 0, V_t = virus concentration in serum (PFU/ml) at time t(1, 5, 10, 30, or 60 min). Three mice per virus were inoculated IV with V3000 (▲, solid line), V3010 (○, solid line), V3012 (△, solid line), V3014 (■, solid line), V3026 (■, dashed line), V3032 (●, dashed line), V3034 (△, dashed line), V3526 (□, dashed line), or V3533 (●, solid line). Each data point is the average of three mice ± SD. The data are representative of two independent assays.

The clearance of infectious virus from the blood was measured after intravenous inoculation (Fig. 4). The viruses that did not interact with cell surface heparan sulfate (V3000, V3034, V3526, and V3533) were cleared very slowly from the blood, less than one order of magnitude decline over 60 min. In contrast, all five viruses that interacted with cell surface heparan sulfate were cleared rapidly (two to five orders of magnitude decline). The mutation at E2 209 from glutamic acid to lysine conferred the most rapid clearance (Fig. 4, V3014 and V3032). As was observed for the infectivity assays, the single-site mutant V3010 and its second-site revertant, V3533, revealed opposite phenotypes. V3010 was cleared rapidly, while V3533, like the parent, was cleared slowly.

The correlation between rapid clearance from the blood and heparan sulfate interaction suggests that virus was removed from the circulation by binding to tissues. This hypothesis was supported by the data in Tables 4 and 5. For all the viruses, infectious virus was found after 60 min in the four organs examined: kidney, liver, lung, and spleen (Table 4). However, the virus titer of an organ represents the virus associated with tissue and the virus in blood perfusing that organ. It was necessary, therefore, to determine whether the virus titer measured for the various organs was due to virus in the blood or virus associated with organ tissue. The organ blood volume can be estimated as 18, 24, 32, and 50% for spleen, kidney, liver, and lung, respectively (Altman and Dittmer, 1971). These values were used to estimate the amount of virus attributable to the blood in each organ. The amount of virus associated with organ tissue was then calculated as the virus titer in the organ minus the virus predicted in the blood fraction. Substantial amounts of infectious virus were found in at least one organ for all nine viruses (Table 5). Virus titration does not account for degraded virus or for virus in the eclipse phase of replication, but these data clearly demonstrate that the rapidly clearing viruses readily bind organ tissue. The virus

TABLE 4

Viral Titers at 60 min after IV Inoculation

VEE clone	Log_{10} (virus titer) PFU/ml or PFU/g (± SD)[a]					
	Serum	Blood	Kidney	Liver	Lung	Spleen
V3000	7.5 (±0.10)	7.3 (±0.12)	7.4 (±0.18)	6.8 (±0.29)	6.9 (±0.11)	6.9 (±0.02)
V3010	5.2 (±0.40)	4.8 (±0.67)	4.7 (±0.18)	3.5 (±0.78)	5.0 (±0.37)	6.4 (±0.01)
V3012	4.9 (±0.03)	4.8 (±0.02)	4.6 (±0.15)	3.3 (±0.49)	4.5 (±0.22)	5.7 (±0.08)
V3014	2.8 (±0.22)	2.5 (±0.17)	3.7 (±0.24)	4.1 (±0.43)	3.5 (±0.17)	4.5 (±0.17)
V3026	4.6 (±0.33)	4.4 (±0.15)	5.1 (±0.28)	3.8 (±1.36)	5.0 (±0.72)	4.9 (±0.91)
V3032	3.2 (±0.84)	3.5 (±0.88)	3.7 (±1.15)	3.2 (±0.86)	4.2 (±0.90)	5.3 (±0.25)
V3034	6.4 (±0.08)	6.3 (±0.20)	6.0 (±0.32)	5.7 (±0.59)	5.7 (±0.11)	5.9 (±0.17)
V3526	5.6 (±0.79)	5.5 (±0.51)	4.2 (±0.31)	4.4 (±0.37)	4.3 (±0.46)	6.0 (±0.14)
V3533	6.9 (±0.28)	6.8 (±0.18)	6.8 (±0.21)	5.9 (±0.90)	6.1 (±0.25)	7.1 (±0.28)

[a] Average of three mice. Data are representative of two independent experiments.

TABLE 5

Amount of Infectious Virus Associated with Organ Tissue and Not Attributable to Virus in Blood Perfusing the Organ

VEE clone	Log_{10} (virus titer associated with organ tissue) PFU/g[a]			
	Kidney	Liver	Lung	Spleen
V3000	7.3	5.6	0[b]	6.5
V3010	4.4	0	4.8	6.4
V3012	4.4	0	3.6	5.7
V3014	3.7	4.2	3.5	4.6
V3026	5.1	4.6	5.3	5.5
V3032	4.5	3.3	4.7	5.3
V3034	5.9	4.2	0	5.6
V3526	0	0	0	5.6
V3533	6.8	4.4	0	7.1

Note. Viral titers were measured at 60 min after an IV inoculation (Table 4).

[a] Virus titer associated with organ tissue was calculated as follows: (virus titer in organ at 60 min) − (virus titer in blood at 60 min × organ blood fraction). Organ blood fraction was estimated as 18, 24, 32, and 50% for spleen, kidney, liver, and lung, respectively (Altman and Dittmer, 1971).

[b] Negative values are reported as zero. Data are the averages for three mice and are representative of two independent experiments.

titers were determined at 60 min after inoculation before viral replication and amplification could occur.

Alternatively, the differences in clearance kinetics could have been explained by three trivial mechanisms. First, the rapidly cleared viruses may have bound to the cell fraction of blood, resulting in serum free of virus. This mechanism was discounted because the serum and whole blood titers for each of the viruses were equivalent at the 60-min time point (Table 4). Note that the whole blood was not treated with anti-coagulants, which can interfere with binding. Second, the viruses may have differed in how well they were recovered from serum or blood. All nine viruses were spiked into serum or whole blood (without anti-coagulant), and the percentage of recovery did not vary significantly (data not shown). Finally, the rapidly cleared viruses may have been less stable. This was examined *in vitro* by spiking serum- and EDTA-treated blood with virus and measuring the virus stability after 1 h at 37°C. All nine viruses were very stable under these conditions with no significant decline in titer (data not shown). Thus, since the recovery and stability of the viruses were similar, we suggest that rapid viral clearance from blood is correlated with efficient binding to GAGs in tissues.

DISCUSSION

Using a cloned epizootic VEE strain and eight site-specific glycoprotein mutants derived from it, we have demonstrated a genetic linkage among cell culture adaptation, use of heparan sulfate as a predominant receptor, attenuation in a mouse model of VEE disease, and acceleration of clearance from the circulation. The virulent parental VEE and three of the mutants did not use HS as a primary receptor in cell culture, suggesting that VEE in nature uses another receptor. Furthermore, those mutants that interacted strongly with HS still infected cells deficient in HS, but with greatly decreased efficiency. This indicates that, under these conditions, even the HS binding mutants may have used the native receptor. Similar results were observed for the related alphavirus, SIN (Byrnes and Griffin, 1998; Klimstra *et al.,* 1998). For VEE mutants selected for efficient growth in cell culture, the ability to bind cell surface HS may function to increase the contact between virus and cell, increasing the probability that the virus will interact with the native receptor. Similar models have been proposed by others (Patel *et al.,* 1993; Spear, 1993; Summerford *et al.,* 1999).

Recent experiments with FMDV (Sa-Carvalho *et al.,* 1997; Baranowski *et al.,* 1998; Escarmis *et al.,* 1998; Neff *et al.,* 1998) and SIN (Klimstra *et al.,* 1998) suggest that HS binding is a tissue culture adaptation, and our data support this hypothesis. The VEE mutants that interacted with HS were selected for rapid binding and penetration on BHK cells (Johnston and Smith, 1988) and, thus, were "tissue culture-adapted." One of the SIN mutants (R114) used by Klimstra *et al.* (Klimstra *et al.,* 1998) was selected in the same manner, and this mutant binds HS. One of the tissue culture-adapted VEE mutants (V3012) has a mutation at the same location as the attenuated VEE vaccine strain, TC-83, which was passaged 83 times in tissue culture (Berge *et al.,* 1961). The mutation is at E2 position 120 from a threonine to a lysine or an arginine for V3012 or TC-83 (Kinney *et al.,* 1989), respectively. In addition, another tissue-culture-adapted VEE mutant (V3010) readily reverts to a non-HS binding phenotype *in vivo,* by a same-site reversion to the V3000 codon or by a second-site reversion from a lysine to glutamate (V3533) (Aronson *et al.,* 2000). This suggests that HS binding is a selective advantage in tissue culture, but not in the vertebrate host. Similar results were observed by Byrnes and Griffin (Byrnes and Griffin, 2000), who isolated SIN mutants upon passage of an HS-binding SIN in mice. These mutants had a net decrease in the charge of E2 and decreased HS binding. In addition, Sa-Carvalho *et al.* (Sa-Carvalho *et al.,* 1997) passed an HS-binding FMDV variant in bovines. The variant reverted to wild type or accumulated a second mutation in a neighboring residue from a lysine to a glutamate.

The tissue culture adaptations that confer HS binding for FMDV, SIN, and VEE are positive charge changes. The FMDV mutations map to the viral surface (Sa-Carvalho *et al.,* 1997; Baranowski *et al.,* 1998; Escarmis *et al.,* 1998), and the positively charged amino acids interact with the negatively charged heparin (Fry *et al.,* 1999) in a conformational rather than a linear domain. For SIN, both conformational and linear heparin-binding sites were

proposed (Klimstra *et al.,* 1998). In contrast, none of the VEE strains used in this study have a linear heparin-binding domain in E2 as defined by others (Cardin and Weintraub, 1989; Sobel *et al.,* 1992; Hileman *et al.,* 1998). Thus, the VEE mutants presumably bind to HS via a conformational domain. The mutations that confer HS binding for SIN (Klimstra *et al.,* 1998) and VEE are located in similar positions on E2, suggesting several regions on alphaviruses that interact with HS. The comparable E2 glycoprotein mutations are: (1) serine to arginine at position 1 for SIN vs glutamate to lysine at positions 3 and 4 for VEE (V3026); (2) glutamate to lysine at position 70 for SIN vs glutamate to lysine at position 76 for VEE (V3010); (3) serine to arginine at position 114 for SIN vs threonine to lysine at position 120 for VEE (V3012). A fourth site was observed for VEE, but not SIN, at position 209 of E2 from a glutamate to a lysine (V3014 and V3032). This site is in a VEE neutralization domain (Johnson *et al.,* 1990) and is exposed on the virion surface.

The importance of region 114 to 120 of E2 is further exemplified by the second-site mutation of V3533—a lysine to a glutamate at position 116. This mutation abrogated HS binding when paired with the mutation at E2 76 (V3010) from a glutamate to a lysine. This suggests that the amino acids at positions 76 and 116 in E2 may be in close proximity on the virion surface and may participate in a conformational HS-binding domain in the mutant V3010, which has a lysine at both positions. In addition, Byrnes and Griffin (Byrnes and Griffin, 2000) have evidence that position 76 of E2 in SIN may act in HS binding. When this amino acid was mutated from a lysine to a glutamate, threonine, or asparagine, HS binding was decreased.

Other VEE strains have positively charged amino acids in the E2 regions proposed to be important in HS binding. The VEE TC-83 vaccine strain has a mutation at E2 position 120 from a threonine to an arginine (Kinney *et al.,* 1989). Two monoclonal antibody resistant mutants of TC-83 have two different mutations in E2—one at position 182 from a serine to an arginine and another at position 199 from a glutamate to a lysine (Johnson *et al.,* 1990). Several investigators have sequenced endemic and epidemic strains of VEE. Two VEE IC strains have two mutations in E2—a glycine to an arginine at position 193 and a threonine to an arginine at position 213 (Wang *et al.,* 1999). An outbreak strain of VEE IAB has a threonine to lysine at position 205 of E2 (Kinney *et al.,* 1992), and a VEE II strain has a lysine at position 209 of E2 (Sneider *et al.,* 1993). All four of these strains were passaged in cell culture prior to sequencing; therefore, it is possible that these mutations resulted from tissue culture adaptation. Mutations that confer HS binding arise very early during passage of SIN on BHK cells (Klimstra *et al.,* 1998).

The ability of the VEE mutants to interact with HS correlated with attenuation in this study. This interaction, however, is not the only determinant of attenuation. One of the non-HS binding mutants (V3526) was completely avirulent. Attenuation of this mutant is likely due to decreased replication *in vivo* (N. L. Davis and R. E. Johnston, unpublished observations). The mechanism for partial attenuation of two other mutants (V3034 and V3533) remains unknown. In another study, Kinney *et al.* (Kinney *et al.,* 1993) mapped the attenuating determinants for the VEE vaccine strain, TC-83, to nucleotide 3 of the 5′ untranslated region and to E2 with the major determinant being amino acid position 120 (Kinney *et al.,* 1993). Spotts *et al.* (Spotts *et al.,* 1998) proposed that one or both of these determinants results in sensitivity to alpha/beta interferons. Our data suggest that HS interaction is a second mechanism of attenuation for TC-83. The VEE mutant, V3012, has a single mutation at E2 position 120 from a threonine to a lysine. This VEE mutant bound HS and was attenuated in adult mice. We predict that TC-83, with the positively charged arginine at E2 position 120, would also interact with HS.

Possible mechanisms of attenuation secondary to HS interaction include inhibition of viral spread and increase in viral clearance. Inhibition of spread from sites of replication may be due to nonproductive binding of virus to HS on the infected cell, adjacent cells, or the extracellular matrix. Clearance from blood may be due to the interaction of virus with HS in various tissues. Viral clearance was examined in this study, and all five VEE mutants that bound HS were rapidly cleared after an intravenous inoculation. Similar results were seen for an HS-binding variant of SIN (Byrnes and Griffin, 2000). In addition, the kinetics of clearance were similar to the clearance of heparin-binding proteins (Wallinder *et al.,* 1984; Karlsson and Marklund, 1988; Yuge *et al.,* 1997), suggesting that rapid clearance was secondary to HS interaction. The lysine at position 209 of E2 conferred the most rapid clearance. This mutation also resulted in the lowest infectivity for the heparan-sulfate-deficient CHO cells, but there was no significant difference between the five HS-binding mutants in the heparin competition and binding experiments. This suggests that the HS-binding VEE mutants differ in their qualitative and/or quantitative binding to HS. Furthermore, the use of heparin in assays does not delineate subtle differences in HS interactions.

Previous alphavirus studies have suggested that rapid clearance is a mechanism of attenuation. Jahrling and Scherer (Jahrling and Scherer, 1973b) found that avirulent strains of VEE, including TC-83, were cleared rapidly from blood, but virulent strains were cleared slowly after intracardiac inoculation of hamsters. The clearance rates were similar to those in this study. The blood titer of the avirulent strains declined two to three orders of magnitude in 60 min, and the blood titer of the virulent strains declined less than one order of magnitude. Similar results were observed in rhesus monkeys (Jahrling *et al.,* 1977a). In another study, six virulent VEE strains were

cleared slowly from the blood of guinea pigs, but only three of six avirulent strains were cleared rapidly (Jahrling *et al.,* 1977b). This suggests, as in this study, that rapid clearance is not the only mechanism of attenuation. Another study used two plaque variants of the VEE IAB Trinidad isolate, the genotypic background of the wild-type virus used in this study (V3000). The benign variant was cleared rapidly from hamster blood, and the virulent variant was cleared slowly (Jahrling and Gorelkin, 1975). Rapid clearance from blood was also correlated with attenuation for other alphaviruses—Western equine encephalitis virus (Jahrling, 1976), Eastern equine encephalitis virus (Marker and Jahrling, 1979), and SIN (Postic *et al.,* 1969; Byrnes and Griffin, 2000). The rapid clearance phenotype was associated with increased binding to cells, agar, or hydroxylapatite (Schleupner *et al.,* 1969; Jahrling and Eddy, 1977; Marker and Jahrling, 1979).

Thus, rapid clearance from blood, secondary to alteration in binding to HS, may play a general role in alphavirus attenuation, resulting in a lower and/or delayed viremia and affecting viral spread throughout the host. Studies are in progress to examine the effects of HS binding on viremia and neuroinvasion.

MATERIAL AND METHODS

Cells

All cells were obtained from the American Tissue Culture Collection. BHK-21 cells (ATCC CCL-10) were maintained in alpha minimal essential medium (Gibco) supplemented with 10% donor calf serum (DCS) and 10% tryptose phosphate broth. The parental CHO-K1 cells (ATCC CRL-61) and GAG-deficient derivatives were maintained in Ham's F-12 medium (Gibco) supplemented with 10% fetal bovine serum. The two GAG-deficient CHO mutants were deficient in all GAGs, pgsA-745 (ATCC CRL-2242) (Esko *et al.,* 1985), or only in heparan sulfate, pgsD-677 (ATCC CRL-2244) (Lidholt *et al.,* 1992).

Viruses

VEE and the site-directed mutant viruses were derived from cDNA clones. The parental virus, V3000, is a molecular clone of the biological 1AB epizootic Trinidad donkey isolate (Davis *et al.,* 1989, 1991). The mutant viruses are isogenic with the parental virus except at defined loci in the glycoproteins (Table 1). The construction of the mutants was described previously (Davis *et al.,* 1991, 1995; Grieder *et al.,* 1995; Aronson *et al.,* 2000). Virus stocks were made by *in vitro* transcription (Ambion) of the cDNA plasmids, electroporation of RNA into BHK cells, and collection of virus in supernatants at 24 h. The electroporation supernatants were used directly without further tissue culture passage, except for the production of radiolabeled virus as described below. The titers of virus stocks were determined by standard plaque assay on BHK cells.

Replicon particles

VEE replicon particles were made using a split helper system as described previously (Pushko *et al.,* 1997). The construction of the replicon genome (V5005) was also described previously (Macdonald and Johnston, 2000). In brief, the replicon genome contained the gene for the green fluorescent protein (GFP) mutant 2 (Cormack *et al.,* 1996) in place of the VEE structural protein genes. The plasmid with GFP mutant 2 was a generous gift from S. Falkow. The replicon genome was packaged in BHK cells by supplying the VEE capsid and glycoproteins on separate helper RNA transcripts. VRP were made with parental or mutant glycoprotein coats by using parental or mutant glycoprotein helpers, respectively. Different VRP are denoted as V5005-3000, V5005-3010, V5005-3014, V5005-3032, V5005-3034, V5005-3526, and V5005-3533, signifying their replicon genome (V5005) and parental or mutant glycoprotein coat (-3000, -3010, etc.). The titers of VRP stocks were determined by counting the number of GFP-expressing BHK cells with a FITC filter set on a fluorescent microscope (Nikon) and were designated as infectious units per milliliter (IU/ml).

VRP infectivity for GAG-deficient CHO cells

CHO-K1, CHO pgsA-745, and CHO pgsD-677 were grown to subconfluency in 24-well tissue culture plates. VRP stocks were diluted in phosphate-buffered saline (PBS), pH 7.4, 1% DCS, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ (PBS diluent), to approximately 2×10^3 IU/ml. The same inoculum (100 μl) was added to triplicate wells of each cell line. The plates were incubated at 37°C, 5% CO_2, for 1 h, medium was added, and the plates were then incubated at 37°C, 5% CO_2, for an additional 18 h. The GFP-expressing cells were counted using a FITC filter set on a fluorescent microscope (Nikon).

Infectivity assays with glycosaminoglycans

Assays to determine the effects of heparin (Sigma), chondroitin sulfate B (Sigma), or dextran sulfate (Sigma) on viral infectivity were performed as described previously (Klimstra *et al.,* 1998). Briefly, 200 PFU of virus was incubated with various concentrations of GAG in PBS diluent for 30 min at 4°C, and then the virus–GAG mixture was incubated with BHK cell monolayers for 60 min at 37°C, 5% CO_2, prior to the addition of an agarose overlay. Plaques were counted after incubation at 37°C, 5% CO_2, for 24 h. The 50% inhibitory concentration was defined as the GAG concentration that inhibited 50% of plaque formation compared to the no-GAG control.

Radiolabeled virus

Virus was radiolabeled with [^{35}S]methionine as described previously (Klimstra *et al.,* 1998). In brief, BHK cells were inoculated with virus stocks at an m.o.i. of 5 to 10, and [^{35}S]methionine was added at 6 h postinoculation. Supernatants were harvested at 24 h postinoculation. The radiolabeled virus was purified, using a 20–60% sucrose discontinuous gradient, followed by a 20–60% sucrose continuous gradient. The virus was concentrated by centrifugation through a 20% sucrose cushion, and the pellets were resuspended in TNE buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA). All sucrose solutions were made weight to volume in TNE buffer.

Virus binding assays

The attachment of radiolabeled virus to heparin–agarose (Sigma) and bovine serum albumin–agarose (Sigma) beads was assessed as described previously (Klimstra *et al.,* 1998). Briefly, radiolabeled virus was diluted in PBS diluent to 1×10^5 CPM/50 μl. Fifty microliters of diluted virus was added to 50 μl of washed beads in duplicate or triplicate. The virus was incubated with the beads for 60 min at 4°C. The beads then were washed three times with 1 ml of PBS diluent and were resuspended in 100 μl of TNE, 0.6% Nonidet P-40. Radioactivity associated with the beads was counted in scintillation fluid using a beta-counter. Elution of bound virus was performed using 50 mM step gradients of NaCl in 10 mM phosphate, pH 7.4, from 100 mM to 1 M NaCl.

Murine studies

Mice were housed in an environmentally controlled room in a BL-3 facility and were given food and water ad libitum. Female, outbred CD-1 mice (Charles River) were obtained at 5 weeks of age and were acclimatized for 1 to 2 weeks. All mice were between 6 and 7 weeks of age at the start of the experiments.

The morbidity and mortality for each virus were determined by inoculating eight mice per group with 10^3 PFU of virus subcutaneously in the left rear footpad. Mice were evaluated clinically and weighed daily for 3 weeks. Observed clinical signs included ruffled fur, paresis, ataxia, hyperaesthesia, and circling. Morbidity was defined as greater than 10% weight loss. A productive infection was confirmed in all surviving mice by a positive antibody titer to VEE by ELISA or by protection from V3000 challenge.

Studies to determine the clearance of virus from the blood were performed as follows. All virus stocks used in the clearance studies were concentrated by centrifugation through a 20% sucrose cushion (weight to volume in TNE). The virus was resuspended in low endotoxin PBS, 1% DCS (animal inoculation buffer), and stored at −80°C. Virus was diluted in animal inoculation buffer immediately prior to inoculation, and the remaining inoculum was stored at −80°C. The titer of each inoculum was confirmed and used to calculate the virus at time zero for each mouse. Three mice per group were inoculated intravenously (IV) in the lateral tail vein with 10^8 PFU of virus in 100 μl. Mice were bled (20–50 μl) at 1, 5, 10, and 30 min postinoculation (p.i.) from the ventral tail artery. At 60 min p.i., the mice were euthanized, and blood, kidney, liver, lung, and spleen were collected. Blood was allowed to clot for 30–60 min at room temperature. Sera were harvested after centrifugation for 10 min at 2000*g*. At the 60-min time point, a portion of the blood sample was not processed for serum and was retained as whole blood. PBS diluent was added to the organs to make a 10% solution (weight to volume). All samples were frozen at −80°C prior to titration on BHK cells. Whole blood and organ samples were homogenized after thawing. The concentration of virus at time zero was calculated for each mouse by dividing the inoculum dose (PFU) by the total blood volume (ml) of each mouse. The total blood volume (ml) was estimated by multiplying the weight (g) of each mouse by 0.07 (Altman and Dittmer, 1971).

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REFERENCES

Altman, P. L., and Dittmer, D. S. (1971). "Respiration and Circulation." Federation of American Societies for Experimental Biology, Bethesda.

Aronson, J. F., Grieder, F. B., Davis, N. L., Charles, P. C., Knott, T. A., Brown, K. W., and Johnston, R. E. (2000). A single-site mutant and revertants arising *in vivo* define early steps in the pathogenesis of Venezuelan equine encephalitis virus. *Virology* **270,** 111–113.

Asagoe, T., Inaba, Y., Jusa, E. R., Kouno, M., Uwatoko, K., and Fukunaga, Y. (1997). Effect of heparin on infection of cells by equine arteritis virus. *J. Vet. Med. Sci.* **59,** 727–728.

Baranowski, E., Sevilla, N., Verdaguer, N., Ruiz-Jarabo, C. M., Beck, E., and Domingo, E. (1998). Multiple virulence determinants of foot-and-mouth disease virus in cell culture. *J. Virol.* **72,** 6362–6372.

Berge, T. O., Banks, I. S., and Tigertt, W. D. (1961). Attenuation of Venezuelan equine encephalomyelitis virus by in vitro cultivation in guinea pig heart cells. *Am. J. Hyg.* **73,** 209–218.

Berger, E. A., Murphy, P. M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annu. Rev. Immunol.* **17,** 657–700.

Byrnes, A. P., and Griffin, D. E. (1998). Binding of Sindbis virus to cell surface heparan sulfate. *J. Virol.* **72,** 7349–7356.

Byrnes, A. P., and Griffin, D. E. (2000). Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. *J. Virol.* **74,** 644–651.

Cardin, A. D., and Weintraub, H. J. R. (1989). Molecular modeling of protein–glycosaminoglycan interactions. *Arteriosclerosis* **9,** 21–32.

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M. (1997). Dengue virus infectivity depends on

envelope protein binding to target cell heparan sulfate. *Nat. Med.* **3,** 866–871.

Chung, C.-S., Hsaio, J.-C., Chang, Y.-S., and Chang, W. (1998). A27L protein mediated vaccinia virus interaction with cell surface heparan sulfate. *J. Virol.* **72,** 1577–1585.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33–38.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312,** 763–767.

Davis, N. L., Brown, K. B., Greenwald, G. F., Zajac, A. J., Zacny, V. L., Smith, J. F., and Johnston, R. E. (1995). Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. *Virology* **212,** 102–110.

Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J., Smith, J. F., and Johnston, R. E. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: Construction of single and multiple mutants in a full-length cDNA clone. *Virology* **183,** 20–31.

Davis, N. L., Willis, L. V., Smith, J. F., and Johnston, R. E. (1989) *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. *Virology* **171,** 189–204.

de la Monte, S., Castro, F., Bonilla, N. J., Gaskin de Urdaneta, A., and Hutchins, G. M. (1985). The systemic pathology of Venezuelan equine encephalitis virus infection in humans. *Am. J. Trop. Med. Hyg.* 194–202.

Escarmis, C., Carrillo, E. C., Ferrer, M., Arriaza, J. F., Lopez, N., Tami, C., Verdaguer, N., Domingo, E., and Franze-Fernandez, M. T. (1998). Rapid selection in modified BHK-21 cells of a foot-and-mouth disease virus variant showing alterations in cell tropism. *J. Virol.* **72,** 10171–10179.

Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985). Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc. Natl. Acad. Sci. USA* **82,** 3197–3201.

France, J. K., Wyrick, B. C., and Trent, D. W. (1979). Biochemical and antigenic comparison of the envelope glycoproteins of Venezuelan equine encephalomyelitis virus strains. *J. Gen. Virol.* **44,** 725–740.

Fry, E. E., Lea, S. M., Jackson, T., Newman, J. W., Ellard, F. M., Blakemore, W. E., Abu-Ghazaleh, R., Samuel, A., King, A. M., and Stuart, D. I. (1999). The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. *EMBO J.* **18,** 543–554.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280,** 1618–1620.

Gleiser, C. A., Gochenour, W. S. Jr., Berge, T. O., and Tigertt, W. D. (1961). The comparative pathology of experimental Venezuelan equine encephalomyelitis virus infection in different animal hosts. *J. Infect. Dis.* **110,** 80–97.

Grieder, F. B., Davis, N. L., Aronson, J. F., Charles, P. C., Sellon, D. C., Suzuki, K., and Johnston, R. E. (1995). Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology* **206,** 994–1006.

Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt, R. J. (1998). Glycosaminoglycan–protein interactions: Definition of consensus sites in glycosaminoglycan binding proteins. *Bioessays* **20,** 156–167.

Ibrahim, J., Griffin, P., Coombe, D. R., Rider, C. C., and James, W. (1999). Cell-surface heparan sulfate facilities human immunodeficiency virus type 1 entry into some cell lines but not primary lymphocytes. *Virus Res.* **60,** 159–169.

Jackson, T., Ellard, F. M., Abu Ghazaleh, R., Brookes, S. M., Blakemore, W. E., Corteyn, A. H., Stuart, D. I., Newman, J. W. I., and King, A. M. Q. (1996). Efficient infection of cells in culture by type O foot and mouth disease virus requires binding to cell surface heparan sulfate. *J. Virol.* **70,** 5282–5287.

Jahrling, P. B. (1976). Virulence heterogeneity of a predominantly avirulent Western equine encephalitis virus population. *J. Gen. Virol.* **32,** 121–128.

Jahrling, P. B., and Eddy, G. A. (1977). Comparisons among members of the Venezuelan encephalitis virus complex using hydroxylapatite column chromatography. *Am. J. Epidemol.* **106,** 408–417.

Jahrling, P. B., and Gorelkin, L. (1975). Selective clearance of a benign clone of Venezuelan equine encephalitis virus from hamster plasma by hepatic reticuloendothelial cells. *J. Infect. Dis.* **132,** 667–676.

Jahrling, P. B., Heisey, G. B., and Hesse, R. A. (1977b). Evaluation of vascular clearance as a marker for virulence of alphaviruses: Disassociation of rapid clearance with low virulence of Venezuelan encephalitis virus strains in guinea pigs. *Infect. Immun.* **17,** 356–360.

Jahrling, P. B., Hilmas, D. E., and Heard, C. D. (1977a). Vascular clearance of Venezuelan equine encephalomyelitis viruses as a correlate to virulence for rhesus monkeys. *Arch. Virol.* **55,** 161–164.

Jahrling, P. B., and Scherer, W. F. (1973a). Histopathology and distribution of viral antigens in hamsters infected with virulent and benign Venezuelan equine encephalitis viruses. *Am. J. Pathol.* **72,** 25–34.

Jahrling, P. B., and Scherer, W. F. (1973b). Growth curves and clearance rates of virulent and benign Venezuelan encephalitis viruses in hamsters. *Infect. Immun.* **8,** 456–462.

Johnson, B. J., Brubaker, J. R., Roehrig, J. T., and Trent, D. W. (1990). Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein. *Virology* **177,** 676–683.

Johnston, R. E., and Smith, J. F. (1988). Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. *Virology* **162,** 437–443.

Joyce, J. G., Tung, J. S., Przysiecki, C. T., Cook, J. C., Lehman, E. D., Sands, J. A., Jansen, K. U., and Keller, P. M. (1999). The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *J. Biol. Chem.* **274,** 5810–5822.

Jusa, E. R., Inaba, Y., Kouno, M., and Hirose, O. (1997). Effect of heparin on infection of cells by porcine reproductive and respiratory syndrome virus. *Am. J. Vet. Res.* **58,** 488–491.

Kari, B., and Gehrz, R. (1992). A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. *J. Virol.* **66,** 1761–1764.

Karlsson, K., and Marklund, S. L. (1988). Plasma clearance of human extracellular-superoxide dismutase C in rabbits. *J. Clin. Invest.* **82,** 762–766.

Kinney, R. M., Chang, G. J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M., and Trent, D. W. (1993). Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5′-noncoding region and the E2 envelope glycoprotein. *J. Virol.* **67,** 1269–1277.

Kinney, R. M., Johnson, B. J., Welch, J. B., Tsuchiya, K. R., and Trent, D. W. (1989). The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. *Virology* **170,** 19–30.

Kinney, R. M., Tsuchiya, K. R., Sneider, J. M., and Trent, D. W. (1992). Molecular evidence for the origin of the widespread Venezuelan equine encephalitis epizootic of 1969 to 1972. *J. Gen. Virol.* **73,** 3301–3305.

Kjellen, L., and Lindahl, U. (1991). Proteoglycans: structures and interactions. *Ann. Rev. Biochem.* **60,** 443–475.

Klimstra, W. B., Ryman, K. D., and Johnston, R. E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J. Virol.* **72,** 7357–7366.

Klimstra, W. B., Ryman, K. D., Nguyen, K. B., Biron, C. A., and Johnston, R. E. (1999). Infection of neonatal mice with Sindbis virus results in a systemic inflammatory response syndrome. *J. Virol.* **73,** 10387–10398.

Krusat, T., and Streckert, H. J. (1997). Heparin-dependent attachment of respiratory syncytial virus (RSV) to cells. *Arch. Virol.* **142,** 1247–1254.

Lidholt, K., Weinke, J. L., Kiser, C. S., Lugemwa, F. N., Bame, K. J.,

Chiefetz, S., Massague, J., Lindahl, U., and Esko, J. D. (1992). A single mutation affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA* **89,** 2267–2271.

Ludwig, G. V., Kondig, J. P., and Smith, J. F. (1996). A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J. Virol.* **70,** 5592–5599.

Macdonald, G. H., and Johnston, R. E. (2000). Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J. Virol.* **74,** 914–922.

Marker, S. C., and Jahrling, P. B. (1979). Correlation between virus–cell receptor properties of alphaviruses in vitro and virulence in vivo. *Arch. Virol.* **62,** 53–62.

Mastromarino, P., Conti, C., Petruzziello, R., Lapadula, R., and Orsi, N. (1991). Effect of polyions on the early events of Sindbis virus infection of Vero cells. *Arch. Virol.* **121,** 19–27.

Mettenleiter, T. C., Zsak, L., Zuckerman, F., Sugg, N., Kern, H., and Ben-Porat, T. (1990). Interaction of glycoprotein gcIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *J. Virol.* **64,** 278–286.

Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87,** 427–436.

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P. W., Blystone, S. D., Brown, E. J., and Baxt, B. (1998). Foot-and-mouth disease virus virulent for cattle utilizes the integrin alpha(v)beta3 as its receptor. *J. Virol.* **72,** 3587–3594.

Neyts, J., Snoeck, R., Schols, D., Balzarini, J., Esko, J. D., Van Schepdael, A., and De Clercq, E. (1992). Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology* **189,** 48–58.

Okazaki, K., Matsuzaki, T., Sugahara, Y., Okada, J., Hasebe, M., Iwamiura, Y., Ohnishi, M., Kanno, T., Shimizu, M., Honda, E., and Kono, Y. (1991). BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. *Virology* **181,** 666–670.

Paredes, A. M., Brown, D. T., Rothnagel, R., Chiu, W., Schoepp, R. J., Johnston, R. E., and Prasad, B. V. (1993). Three-dimensional structure of a membrane-containing virus. *Proc. Natl. Acad. Sci. USA* **90,** 9095–9099.

Patel, M., Yanagishita, M., Roderiquez, G., Bouhabib, D. C., Oravecz, T., Hascall, V. C., and Norcross, M. A. (1993). Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. *AIDS Res. Hum. Retroviruses* **9,** 167–174.

Postic, B., Schleupner, C. J., Armstrong, J. A., and Ho, M. (1969). Two variants of Sindbis virus which differ in interferon induction and serum clearance. I. The phenomenon. *J. Infect. Dis.* **120,** 339–347.

Pushko, P., Parker, M., Ludwig, G. V., Davis, N. L., Johnston, R. E., and Smith, J. F. (1997). Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: Expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* **239,** 389–401.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V., and Srivastava, A. (1999). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2 [see comments]. *Nat. Med.* **5,** 71–77.

Rice, C. M., and Strauss, J. H. (1982). Association of sindbis virion glycoproteins and their precursors. *J. Mol. Biol.* **154,** 325–348.

Roehrig, J. T., Hunt, A. R., Kinney, R. M., and Mathews, J. H. (1988). In vitro mechanisms of monoclonal antibody neutralization of alphaviruses. *Virology* **165,** 66–73.

Sa-Carvalho, D., Reider, E., Baxt, B., Rodarte, R., Tanuri, A., and Mason, P. (1997). Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. *J. Virol.* **71,** 5115–5123.

Schleupner, C. J., Postic, B., Armstrong, J. A., Atchison, R. W., and Ho, M. (1969). Two variants of Sindbis virus which differ in interferon induction and serum clearance. II. Virological characterizations. *J. Infect. Dis.* **120,** 348–355.

Sneider, J. M., Kinney, R. M., Tsuchiya, K. R., and Trent, D. W. (1993). Molecular evidence that epizootic Venezuelan equine encephalitis (VEE) I-AB viruses are not evolutionary derivatives of enzootic vee subtype I-E or II viruses. *J. Gen. Virol.* **74,** 519–523.

Sobel, M., Soler, D. F., Kermode, J. C., and Harris, R. B. (1992). Localization and characterization of a heparin binding domain peptide of human von Willebrand factor. *J. Biol. Chem.* **267,** 8857–8862.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Semin. Virol.* **4,** 167–180.

Spotts, D. R., Reich, R. M., Kalkhan, M. A., Kinney, R. M., and Roehrig, J. T. (1998). Resistance to alpha/beta interferons correlates with the epizootic and virulence potential of Venezuelan equine encephalitis viruses and is determined by the 5′ noncoding region and glycoproteins. *J. Virol.* **72,** 10286–10291.

Summerford, C., Bartlett, J. S., and Samulski, R. J. (1999). AlphaVbeta5 integrin: A co-receptor for adeno-associated virus type 2 infection [see comments]. *Nat. Med.* **5,** 78–82.

Summerford, C., and Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72,** 1438–1445.

Vanderplasschen, A., Bublot, M., Dubuisson, J., Pastoret, P. P., and Thiry, E. (1993). Attachment of the gammaherpesvirus bovine herpesvirus 4 is mediated by the interaction of gp8 glycoprotein with heparinlike moieties on the cell surface. *Virology* **196,** 232–240.

Victor, J., Smith, D. G., and Pollack, A. D. (1956). The comparative pathology of Venezuelan equine encephalomyelitis. *J. Infect. Dis.* **98,** 55–66.

Wallinder, L., Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona, G. (1984). Hepatic and extrahepatic uptake of intravenously injected lipoprotein lipase. *Biochim. Biophys. Acta* **795,** 513–524.

Wang, E., Barrera, R., Boshell, J., Ferro, C., Freier, J. E., Navarro, J. C., Salas, R., Vasquez, C., and Weaver, S. C. (1999). Genetic and phenotypic changes accompanying the emergence of epizootic subtype IC Venezuelan equine encephalitis viruses from an enzootic subtype ID progenitor. *J. Virol.* **73,** 4266–4271.

Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S., and Strauss, J. H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J. Virol.* **66,** 4992–5001.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H., and Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246,** 179–189.

Weaver, S. C. (1986). Electron microscopic analysis of infection patterns for Venezuelan equine encephalomyelitis virus in the vector mosquito, Culex (Melanoconion) taeniopus. *Am. J. Trop. Med. Hyg.* **35,** 624–631.

WuDunn, D., and Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63,** 52–58.

Yuge, T., Furukawa, A., Nakamura, K., Nagashima, Y., Shinozaki, K., Nakamura, T., and Kimura, R. (1997). Metabolism of the intravenously administered recombinant human basic fibroblast growth factor, trafermin, in liver and kidney: Degradation implicated in its selective localization to the fenestrated type microvasculatures. *Biol. Pharmaceut. Bull.* **20,** 786–793.

Zhou, F., Hook, T., Thompson, J. A., and Hook, M. (1992). Heparin protein interactions. *Adv. Exp. Med. Biol.* **313,** 141–153.